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13. ABSTRACT (Maximum 200 Words) One of the most frequent alterations in breast cancer is deregulation of the p53 tumor suppression signaling pathways. The tumor suppressor p53 is a sequence-specific transcription factor that is activated in response to various cellular stresses. It has been predicted that there are hundreds of consensus p53 binding sites present in the human genome. To date, numerous seminal p53 target genes have been identified, leading to the further elucidation of the role of p53 in tumor suppression. However, only a subset of p53 target genes has been identified to date, and characterization of p53 signaling pathways in their entirety is not yet complete. The goal of this study is to further define and characterize such pathways through the identification of novel genes that are directly regulated by p53. Using chromatin immunoprecipitation followed by a yeast selection system we have isolated over 100 genomic DNA fragments that contain novel p53 binding sites. The new DNA fragments obtained have been mapped to various regions of the human genome, and putative novel p53 target genes have been identified, validated, and characterization is ongoing. This research will lead to a more complete understanding of p53-regulated signaling pathways in mammary epithelial cells.				
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INTRODUCTION

As stated in my original grant proposal, the overall goal of my project was to explore the pivotal role that the transcription factor p53 plays in suppressing neoplastic transformation of the normal mammary epithelium. Specifically, I proposed to study p53 signaling pathways in primary mammary epithelial cells in response to oncogenic stress as compared to genotoxic stress. The following hypothesis was put forth. **p53 protein levels in the cell and the heterogeneity of the p53 consensus DNA binding site contained within downstream target genes dictate p53 target gene selectivity in normal human mammary epithelial cells (HMECs) after an oncogenic stimulus.**

BODY

The hypothesis for the proposed studies was based on observations from other laboratories indicating that overexpression of oncogenes (such as myc and ras) would activate p53. ErbB2 is an oncogene found to be overexpressed in 20-30% of primary human breast tumors (1,2), and this was the oncogene of choice for our studies due to its physiological relevance. However, upon ectopic expression of ErbB2 in primary human mammary epithelial cells, we found that p53 protein levels decreased and target gene induction was not seen. Our data was subsequently supported by a paper from Mien-Chie Hung's group in which they showed in NIH3T3 cells that overexpression of ErbB2 induces p53 ubiquitination via phosphorylation of Mdm2 by Akt (3). Mdm2 is an E3 ubiquitin ligase protein that negatively regulates p53 through ubiquitination and subsequent degradation (4, 5). Phosphorylation of Mdm2 by Akt leads to Mdm2 activation and decreased protein levels of p53 (3). The original intent of this project was to compare the p53 binding sites and target genes isolated in response to oncogenic stress versus genotoxic stress. In light of the above-mentioned oncogene inconsistencies, my advisor and Ph.D. Committee recommended that I reprioritize my project to pursue the proposed genotoxic stress studies as a primary objective and move the oncogene-based research to a secondary objective. Thus, I will be reporting primarily on the work that I've completed for my original Statement of Work with regards to genotoxic stress.

Task 1. To determine the kinetics of p53 binding to select target genes, quantify p53 protein levels, and assess the phosphorylation and acetylation status of p53 *in vivo* in human mammary epithelial cells after oncogenic stimulation.

- a. Obtain normal human mammary epithelial cells and mammary epithelial cell lines to be used in the following experiments and verify p53 status (Months 1-3). **COMPLETED**
 - A purification process for primary human mammary epithelial cells (HMECs) from reduction mammoplasty breast tissue as well as the growth conditions for these cells have been optimized. Experiments have been completed that determined the p53 status in each sample. All samples obtained to date contain functional p53 in response to genotoxic stress (Figure 1).
- b-e. Due to the inconsistencies experienced with regards to the activation of p53 by the ErbB2 protein (discussed above) we have not been able to proceed with tasks b-e. Instead, we have focused on the Task 2 objectives.

Task 2. To “trap” p53 *in vivo* at consensus DNA binding sites after oncogenic stress, and analyze: (i) the heterogeneity of the sites to which p53 is bound compared to the canonical binding site, (ii) the affinity of p53 for a select number of the DNA binding sites recovered, and (iii) the genetic loci and adjacent coding region for novel sites.

- a. Create libraries of DNA fragments to which p53 binds in response to oncogenic stimulation and genotoxic stress by cloning the fragments recovered from chromatin immunoprecipitation into a yeast reporter vector. (Months 6-12).
 - To date we have generated three libraries from different cell cultures. The first library generated was from MCF-10A cells, an immortal but nontransformed mammary epithelial cell line; the second library was generated from the primary human mammary epithelial cells (HMECs), and the third library was generated from the MCF-7 breast cancer cell line. All cells used contain functional p53. The p53-activating treatment was 350 μ M adriamycin for 6 h. Adriamycin (doxorubicin) is a commonly used breast cancer chemotherapeutic agent.
- b-c. Use a functional yeast selection system to identify p53 DNA binding sites that promote transcriptional activation of a reporter gene, and sequence the DNA fragments recovered from the yeast screen (Months 12-18).
 - The yeast screen of the first MCF-10A library has been completed. We have identified over 100 novel p53 binding sites. In addition we have also recovered p53 binding sites of previously identified p53 target genes, including Mdm2 (Table 1).
 - The yeast screen of the HMEC library is in progress. To date we have identified numerous novel p53 binding sites as well as the known p53 binding site present in the p21 promoter. p21 is a well-characterized p53 target gene that promotes cell cycle arrest.
 - The yeast screen of the MCF-7 library has just been initiated and thus, binding sites will be recovered within the next 2 months.
- d. Test p53-binding DNA fragments identified in (b) in a mammalian cell reporter assay (Months 12-24).
 - Data for this task has not been generated to date, although we anticipate the reporter assay analysis occurring on schedule within the next 12 months.
- e. Compare the characteristics and heterogeneity of the newly identified p53 binding sites that were isolated from mammary epithelial cells subjected to oncogenic stress versus genotoxic stress (Months 24-36).
 - These issues have not been addressed yet.
- f. Test the affinity of p53 for the newly identified binding sites by employing an *in vitro* binding assay (Month 12-24).

- Data for this task has not been generated to date, although we anticipate the *in vitro* analysis occurring on schedule within the next 12 months.

g. Use genome sequence resources to identify candidate genes that may be directly regulated by p53 binding to the novel sites identified, and verify p53-dependent gene transactivation of these candidate genes through Northern analysis (Months 12-36).

- A candidate gene is defined as a gene that is located within 20 kb from where the fragment that has been isolated from chromatin immunoprecipitation aligns in the human genome. Candidate genes have been identified from the MCF-10A library and the HMEC library, although the HMEC library list is not complete because the screening of this library is ongoing. We have generated primers to over 40 candidate genes for the MCF-10A library, and are currently analyzing these genes by Northern and RT-PCR to determine p53 regulation of gene expression.
- A model system has been used to validate the putative p53 target genes for the MCF-10A library. A stable MCF-10A cell line expressing the Human Papilloma Virus 16 E6 (MCF-10A-E6) protein has been developed, as well as the empty vector control cell line (MCF-10A-Neo). In the presence of the E6 viral protein, p53 is rendered nonfunctional due to E6 binding and subsequent degradation of the p53 protein. Thus, when MCF-10A-E6 cells are treated with adriamycin (350 μ M) for 8 h, there is no induction of p53 protein levels or of p21 protein, a known p53 target gene. However, in the MCF-10A-Neo cells there is a marked induction of p53 and p21 (Figure 2). These two cell lines are being used in Northern analysis and RT-PCR to validate the p53 dependence of the candidate gene regulation.
- Because the HMEC cells are primary and do not undergo transfection efficiently, we have turned to a retroviral method to generate a model validation system for genes identified from the HMEC library. We have seen that HMEC cells infect at high efficiency when using a retroviral vector expressing the ErbB2 gene. Thus, we have decided to employ this very efficient retroviral infection protocol to express a p53 small interfering RNA (siRNA) construct. p53 siRNA should "knock-down" the amount of p53 mRNA in the cells which will result in less protein and little/no p53 activity.

KEY RESEARCH ACCOMPLISHMENTS

- Characterized HMECs with regards to optimal growth conditions and p53 status.
- Generated chromatin immunoprecipitation libraries from primary epithelial cells as well as from 2 mammary epithelial cell lines, one nontransformed and one transformed.
- Completed yeast screen of MCF-10A library.
- Generated MCF-10A-Neo and MCF-10A-E6 cell lines to be used for target gene validation.

REPORTABLE OUTCOMES

- Poster presentation: Vanderbilt-Ingram Cancer Center Retreat
Nashville, TN
June 6, 2003

title: p53 Signaling in Mammary Epithelial Cells

*received Breast Cancer Program Poster Award

- Poster presentation: American Association for Cancer Research
Advances in Breast Cancer Research
Huntington Beach, CA
October 8-12, 2003

title: Identification of Novel p53 Target Genes in Mammary Epithelial Cells

CONCLUSIONS

The overall goal of this research project is to further elucidate p53 signaling pathways in mammary epithelial cells by identifying novel p53 target genes. The results described above show that novel p53 binding sites are being isolated using our experimental approach. Although the analysis of these binding sites and potential candidate genes is ongoing our preliminary data suggests that we are recovering p53 binding sites that are physiologically relevant, being that we've isolated numerous binding sites from known p53 target genes. The new p53 binding sites obtained have been mapped to various regions of the human genome, and putative novel p53 target genes have been identified, validated, and characterization is ongoing. Although the goals of the research project have been reprioritized with regards to the oncogenic and genotoxic studies, the data obtained from the genotoxic stress portion is itself very valuable and will lead to a more complete understanding of p53-regulated signaling pathways in mammary epithelial cells and how deregulation of these pathways can lead to breast cancer.

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APPENDIX

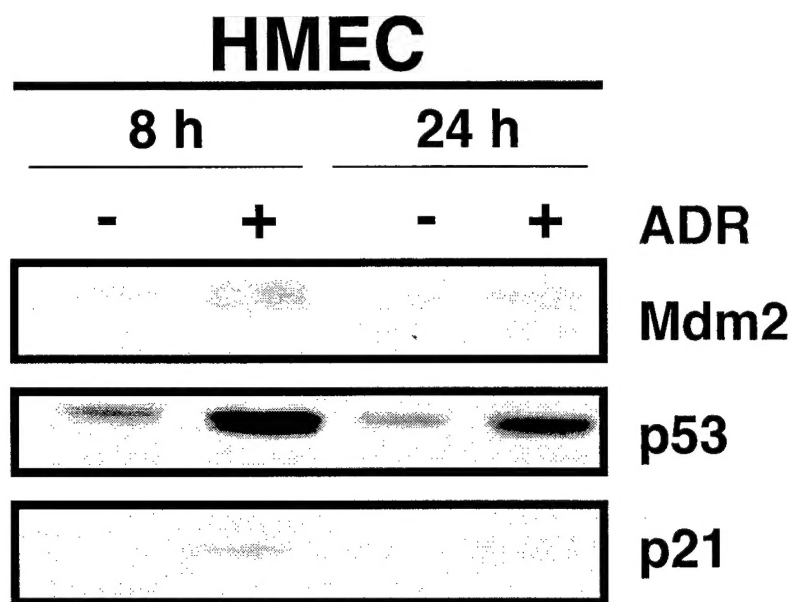


Figure 1. p53 Activation in Primary Human Mammary Epithelial Cells Treated with Adriamycin. Primary HMECs were treated with 350 μ M adriamycin for 8 and 24 h before being harvested and analyzed by Western for p53 protein and the p53 target genes Mdm2 and p21.

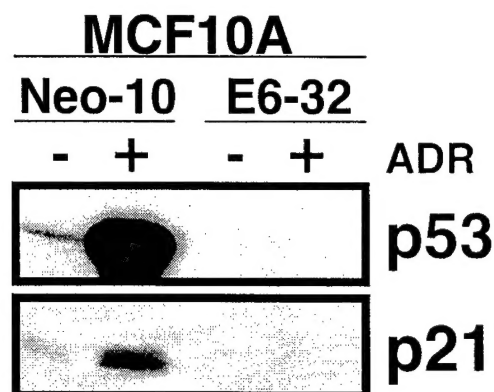


Figure 2. p53 Status of MCF10A-Neo and MCF10A-E6 Cells. MCF10A cells were transfected with the pCMV-NEO or pCMV-HPV16E6 vector and selected clones were analyzed for p53 and p21 induction in response to treatment with 350 μ M adriamycin for 8 h. Clone 10 for the pCMV-Neo transfected and clone 32 for the pCMV-HPV16E6 were used for further candidate target gene validation.

	Binding Site	# of bp matching consensus
p53 consensus	RRRCATGYYY---0-13bp--RRRCATGYYY	
p21	GAACATGTCC----0bp----CAACATGTTG	18/20
Mdm2	GGTCAAGTTG----0bp----GGACACGTCC	17/20
p48	AAACATGCCC----1bp----GGGCTTGTTT	20/20
	AGGCATGTGC----0bp----GAACATGCCT	19/20
	GAGCTTGCCT----1bp----AGACATGCCT	20/20
	GGGCAAGTCT----0bp----GAACATGACC	19/20
	GTGCATGTCC----0bp----AGGCATGTGT	18/20
	GAACATGTAA----0bp----GGACAAGCCA	17/20
	TGACAAGTCT----0bp----GGGCTTGCTC	19/20
	CCACATGCCC----0bp----GGGCAAGCCC	18/20
	GTACATGTCA----0bp----GGGCATGTTG	17/20
	TGACAAGTCT----0bp----GGGCTTGGTC	18/20
	GGGCATGCCA----0bp----GGACATGTCT	19/20

Table 1. p53 binding sites recovered from the MCF10A and HMEC libraries. A small subset of the binding sites recovered from the MCF10A and HMEC libraries are shown compared to the p53 consensus binding site. Also shown are the number of base pair matches to the consensus sequence. Three previously identified p53 target gene binding sites have been identified from our screen and are shown. R= purine, Y=pyrimidine.